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TRANSSTADIAL AND HORIZONTAL TRANSMISSION OF RIFT VALLEY FEVER VIRUS IN *HYALOMMA TRUNCATUM*

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Abstract. We exposed *Hyalomma truncatum* and *Rhipicephalus appendiculatus* to Rift Valley fever (RVF) virus in order to assess the possible role of these ticks as enzootic/epizootic RVF vectors. The virus replicated in *H. truncatum* after intracoelomic inoculation, and a minimum transmission rate of 17% was achieved after 15 days intrinsic incubation. The virus persisted at least 58 days in these ticks. Virus was also shown to pass transstadially from inoculated *H. truncatum* nymphs to adults, with peak viral titers reaching $10^{3.5}$ plaque-forming units (PFU) in adult males after they were provided with bloodmeals. Virus was recovered from adult females 121 days after they were inoculated as nymphs. Viral titers peaked in inoculated male ticks after dropping off a host (mean titer = $10^{4.3}$ PFU). RVF virus was not detected in pools of eggs and larval progeny from 11 infected female *H. truncatum*. *H. truncatum* larvae and nymphs did not become infected after ingesting > $10^{2.0}$ PFU while feeding on a RVF viremic hamster. The number of infected specimens declined rapidly after RVF virus was inoculated into *R. appendiculatus* adults, and virus was undetectable 12 days post-inoculation.

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Rift Valley fever (RVF) virus is transmitted by various species of mosquitoes during epizootics[1–4] and epidemics,[5] and may be maintained during interepizootic periods in Kenya by vertical transmission in ground pool *Aedes* mosquitoes.[6] Prior to 1977, epizootics of RVF were believed to be confined to sub-Saharan Africa.[7] However, the epizootics/epidemics in Egypt in 1977[8] and in Mauritania in 1987[9, 10] occurred in areas outside the normal range of the disease. The method by which the virus was introduced into these areas is unknown.

Evidence suggests that infected mosquitoes and viremic vertebrates may not have been involved in the initial introduction of the virus from Sudan north across the Sahara into Egypt in 1977[11] and from southern Mauritania[12,13] to southwestern Mauritania in 1987.[14] Infected ticks could serve as a possible host for RVF virus, as they can be transported over long distances on their vertebrate hosts. Although very limited field[15] and experimental[16,17] studies have evaluated the potential for ticks to be involved in the ecology of the disease, an isolation of RVF virus was made from one *Amblyomma variegatum* in the Central African Republic. Attempts to isolate RVF virus from ticks during epizootics have not been reported.

Many species of ticks regularly parasitize cattle, sheep, and goats in RVF virus enzootic/epizootic areas in sub-saharan Africa. However, *H. truncatum* is unique in that it is widespread and common throughout the entire geographic range of RVF virus. *H. truncatum* is found on domestic animals that are known to be amplifying hosts of RVF virus, such as cattle, sheep, and goats, and has been implicated as a reservoir and vector of other pathogenic viruses. Although previous studies[16, 17] suggested that *R. appendiculatus* could not become experimentally infected with RVF virus, we studied this species to further examine its potential for virus infection and transmission and to compare its susceptibility to infection to that of *H. truncatum*.

MATERIALS AND METHODS

Ticks, virus, and virus assay procedures

H. truncatum used in this study were established from a colony at the National Institute of Virology, Republic of South Africa. The *R. appendiculatus* were established from the progeny of females collected from cattle in Rift Valley Province, Kenya. The parental generation of ticks used in these studies were tested for the presence of arboviruses by assay in suckling mice and in

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SW-13 and Vero cell monolayers. Only progeny of virus-negative adults were used to establish our colony. Colonies were held in a chamber maintained at 92–98% RH, 26°C with a 12:12 hr L:D photoperiod. The chamber was kept in a BL3 laboratory specifically modified to contain ticks. The ZH501 strain[8] of RVF virus used in these studies was isolated from the serum of a fatal human hemorrhagic fever case in Egypt and passed twice in fetal rhesus lung cells before use in this study.[18]

For virus assays, ticks were triturated in 1 ml (nymphs, flat adults, and pools of eggs or larvae) or 3 ml (individual engorged adults) of 10% fetal bovine serum in Medium 199 with Hanks' salts plus antibiotics. All tick suspensions were then centrifuged at 1,000 × *g* for 10 min at 4°C and passed through a 0.45 μm millipore filter prior to storage at −70°C. Suspensions were later assayed for virus by plaque assay on 2–3-day-old Vero cell monolayers.[19]

Infection of ticks with RVF virus

In Exp. 1, 2, and 3, ticks were infected by intracoelomic (IC) inoculation of ≈0.14–0.27 μl of RVF virus suspension containing $10^{2.7}$ plaque-forming units (PFU) of virus through the membrane between the coxae and trochanter of the fourth leg with a glass microcapillary tube modified to form a fine needle. In each experiment, 10 ticks (5 female, 5 male for Exp. 1 and 2) were sampled at predetermined intervals post-inoculation and stored at −70°C until assayed for RVF virus. Groups of ticks were allowed to feed on either a guinea pig or a hamster at arbitrarily selected intervals post-inoculation (6 and/or 15 days post-inoculation). Although hamsters were the host animal of choice to demonstrate transmission,[19] ticks would only infrequently attach to hamsters, while they readily fed on guinea pigs. Samples of the ticks were collected and stored at −70°C at the day of infestation, at partial engorgement, at drop-off, or after oviposition. Surviving hamsters were challenged with 10^5 PFU of RVF virus 1–2 weeks after tick drop-off to determine if they were susceptible to the virus. Livers from hamsters that died after serving as hosts for ticks were triturated in a Ten Broeck tissue grinder and assayed for virus as described above. Virus was identified as RVF by antigen-capture ELISA.[20] Since RVF virus is normally not fatal to guinea pigs, an indirect fluorescent antibody test[21] was performed on blood samples before tick infestation and again 4–6 weeks after tick drop-off to determine if the ticks had transmitted the virus to the guinea pigs.

In Exp. 1, ≈300 unfed *R. appendiculatus* adults were inoculated with virus. On day 6 post-inoculation, 15 pairs of ticks were allowed to feed on a guinea pig.

In Exp. 2, ≈400 unfed *H. truncatum* adults were inoculated with virus. On day 6 post-inoculation, 15 pairs of ticks were allowed to feed on a guinea pig. On day 15 post-inoculation, 20 female and 15 male ticks were allowed to feed on a guinea pig, and 1 pair of ticks was allowed to feed on each of 9 hamsters.

In Exp. 3, ≈200 unfed *H. truncatum* nymphs were inoculated with virus. On day 6 post-inoculation, a pool of nymphs were allowed to feed on a guinea pig. These nymphs molted to adults on day 44 post-inoculation. Adults were subsequently allowed to feed on another guinea pig on day 57 post-inoculation. In all experiments, progeny of infected females were assayed for virus after we pooled eggs or larvae.

In Exp. 4 and 5, *H. truncatum* larvae and nymphs, respectively, were allowed to feed on viremic hamsters. About 500 larvae were placed on each of 2 hamsters in Exp. 4. In Exp. 5, 20 nymphs were placed on each of 2 hamsters. To synchronize peak viremias in hamsters with the time of maximum tick feeding, hamsters in both experiments were inoculated sc with ≈10^5 PFU of RVF virus 24 hr prior to estimated tick drop-off. After such an exposure, hamsters rapidly develop a viremia of > 10^7 PFU/ml of blood within 16 hr.[19] A sample of ticks in each experiment was assayed for virus at drop-off and after selected periods of incubation. In Exp. 4, after the molt to nymphs, 10 specimens infested each of 8 hamsters. Replete nymphs were assayed for virus at drop-off. In Exp. 5, after the molt to adults, 1 pair of ticks was allowed to feed on each of 7 hamsters. Replete adults were assayed for virus at drop-off. Engorged adult females were not allowed to oviposit. After tick exposure, hamsters serving as hosts were treated as described for Exp. 1–3.

RESULTS

Infection of ticks inoculated with RVF virus

In Exp. 1, 19 days after *R. appendiculatus* adults were inoculated with ≈$10^{2.8}$ PFU RVF virus, viral titers declined to undetectable levels. Virus

was recovered from 10–30% (1–3/10) ticks on days 1–12 post-inoculation. Specimens fed on a guinea pig on day 6 post-inoculation contained no detectable virus at drop-off on day 13 post-inoculation (6 males, 6 females) or after oviposition at day 35 post-inoculation (6 males, 9 females). The host guinea pig sera did not contain detectable RVF antibody when tested 34 days after tick drop-off, sufficient time to develop an antibody response.

In Exp. 2, *H. truncatum* adults were inoculated with $\approx 10^{2.6}$ PFU RVF virus (Fig. 1). Viral titers in unfed specimens initially declined below inoculation levels, but subsequently increased to corresponding levels which were maintained until the experiment was concluded at 51 days post-inoculation (Fig. 1A). Infection rates remained relatively constant from days 1–51 post-inoculation, with an overall infection rate of 81% (106/131). Males fed on day 6 post-inoculation had viral titers after drop-off that were significantly higher (t-test, $P < 0.05$) than inoculation levels (data not shown). Four females fed on day 6 post-inoculation had no detectable virus at drop-off (day 14 post-inoculation), but 4/6 had detectable virus post-oviposition (day 49 post-inoculation). When males and females were fed on day 15 post-inoculation on a guinea pig, viral titers did not differ significantly from unfed specimens (Fig. 1B). None of 35 ticks feeding on guinea pigs at days 6 or 15 post-inoculation transmitted virus. The progeny of 7 infected females that fed on guinea pigs were tested for virus by assaying a pool of ≈ 200 flat larvae. No virus was recovered from these ticks. When a pair of ticks infested each of 9 hamsters on day 15 post-inoculation, 3 of the hamsters died in 4–6 days. The minimum transmission rate was 17% (3/18). Rift Valley fever virus was isolated and identified from the livers of the 3 dead hamsters. The 6 hamsters that survived tick feeding were challenged with 10^5 PFU of RVF virus and all died within 36 hr, thus demonstrating their susceptibility to the virus.

Viral titers in *H. truncatum* nymphs inoculated in Exp. 3 with $10^{2.7}$ PFU RVF virus did not increase, even after feeding on day 14 (Fig. 2). The virus was detected in 8/10 (80%) fed nymphs and in 7/10 (70%) adults tested after molting on day 57 post-inoculation. The viral titers of males increased significantly after feeding (days 60 and 69 post-inoculation). When tested after feeding, 9/11 (81.8%) males and 5/17 (29.4%) females were infected. Overall, 21/38 (55.3%) adults were

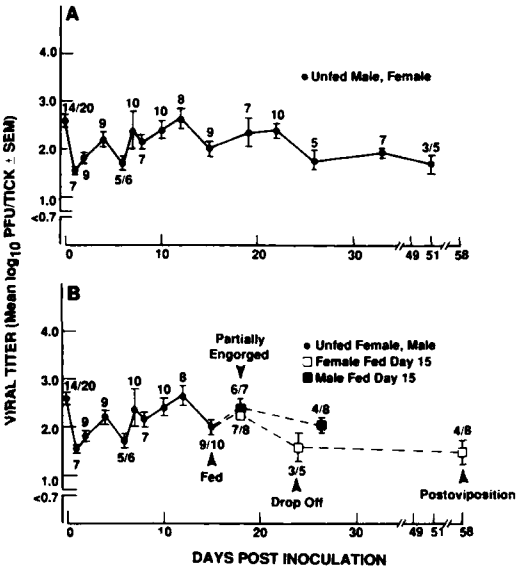

FIGURE 1. Rift Valley fever viral titers in intracoelomically inoculated *Hyalomma truncatum* that were unfed **(A)** or fed at 15 days post-inoculation **(B)**. The mean viral titer (±SE) of all ticks with detectable virus is shown for each sampling date, except when all specimens contained undetectable virus ($< 10^{0.7}$ PFU). Unless otherwise noted, 10 specimens were tested for virus at each sampling date; the number found to be virus positive is shown.

transstadially infected, including 14/16 (87.5%) males and 7/22 (31.8%) females. Nymphs and adults that fed on guinea pigs did not transmit the virus. Virus was not detected in the eggs from 1 infected female and the progeny larvae from 3 other infected females.

Infected of ticks orally exposed

Attempts to orally infect *H. truncatum* with RVF virus in Exp. 4 and 5 were unsuccessful. Both larvae (13/20; mean titer = $10^{2.7}$ PFU/tick) and nymphs (9/10; mean titer = $10^{2.3}$ PFU/tick) ingested virus while feeding on viremic hamsters. In Exp. 4, fed larvae did not contain detectable virus when tested 5 days after drop-off ($n = 20$), and no virus was detected in these ticks after the nymphal molt either before ($n = 20$) or after ($n = 20$) bloodfeeding. Nymphs that were fed on uninfected hamsters day 16 after drop-off from a viremic host did not transmit virus to hamsters. In Exp. 5, nymphs ($n = 5$) tested 12 days after a viremic bloodmeal did not contain detectable virus. The subsequent adults did not contain detectable virus after feeding on a ham-

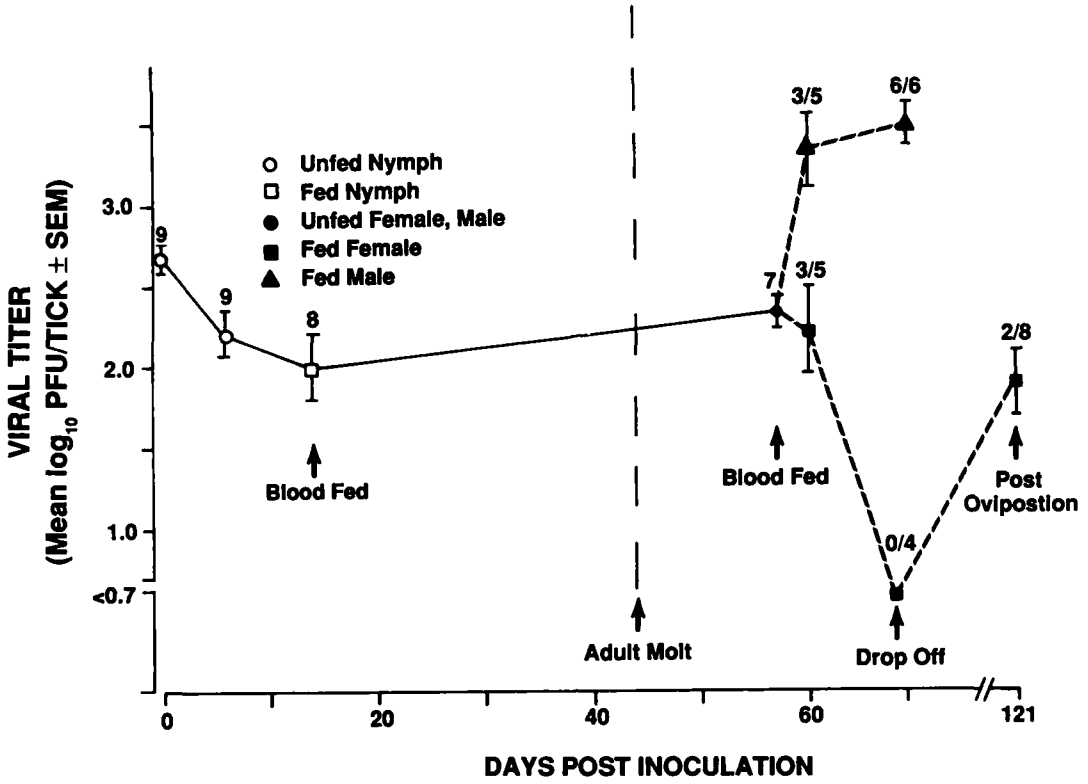

FIGURE 2. Rift Valley fever viral titers in *Hyalomma truncatum* infected as nymphs by intracoelomic inoculation. The mean viral titer (±SE) of all ticks with detectable virus is shown for each sampling date, except when all specimens contained undetectable virus ($<10^{0.7}$ PFU). Unless otherwise noted, 10 specimens were tested for virus at each sampling date; the number found to be virus positive is shown.

ster 45 days after drop-off from a viremic host, and did not transmit the virus to the hamsters.

DISCUSSION

We believe this study to be the first reported demonstration of RVF viral replication in and transmission by ticks. Rift Valley fever virus titers increased almost 50-fold (mean titer = $10^{4.3}$ PFU/tick) in fed male *H. truncatum* ticks after IC inoculation in Exp. 2. Infected adult ticks transmitted virus to hamsters after 15 days of extrinsic incubation. However, ticks did not transmit RVF virus while engorging on guinea pigs. Post-oviposition females retained detectable virus for at least 58 days after inoculation. The virus persisted in unfed males and females without a significant change in titer for at least 51 days post-inoculation. Virus titers increased above IC inoculation levels in fed *H. truncatum* males which had originally been infected as nymphs, but neither nymphs nor adults transmitted virus to guinea pigs. In contrast, RVF virus apparently did not replicate in inoculated *R. appendiculatus* adults in Exp. 1. Thirty percent of these ticks retained the virus for up to 12 days after inoculation, and bloodfeeding did not enhance viral replication. Daubney and Hudson[1] reported that *R. appendiculatus* nymphs orally exposed to RVF virus could retain the virus for up to 7 days after exposure, but lost the virus during the molt to adult.

No evidence of vertical transmission of RVF virus was found in *H. truncatum* in Exp. 2. However, the progeny groups from only 11 females (7 inoculated as adults, 4 inoculated as nymphs) were tested in this study. The progeny from a larger number of infected females must be tested to determine the potential for vertical passage of RVF virus in this species. Rates of vertical transmission for central European encephalitis virus in *Ixodes ricinus* are reported to be as low as 3.3%.[22] Because we tested all progeny from the 11 selected parental females, the occurrence of

low filial infection rates within progeny groups, like those reported by Singh and others[23] for Kyasanur Forest disease in *Ixodes petauristae,* would not have interfered with our ability to detect vertical transmission of the virus.

We report transstadial transmission of RVF virus for what we believe is the first time between inoculated *H. truncatum* nymphs and the subsequent adults (Exp. 3). The rate of transstadial transmission (percent infected adults/percent infected nymphs) was 63%. The virus was found in post-oviposition females for at least 121 days after the inoculation of nymphs.

Although *H. truncatum* was a competent vector of RVF virus after IC inoculation, capable both of transstadial and horizontal transmission to hamsters, it was not a competent vector in the laboratory after the ingestion of virus from viremic hosts. The virus did not replicate in *H. truncatum* larvae and nymphs which had ingested an average of $10^{2.7}$ and $10^{2.3}$ PFU of virus, respectively (Exp. 4 and 5). No virus was detected in orally exposed ticks tested after drop-off from the viremic hamster. These results are similar to those reported for 5 species of ixodid ticks[16] and 1 species of argasid tick[17] orally exposed to RVF virus. It is possible that the amount of virus ingested was below the infection threshold of this species, although the very high viremias developed by hamsters (comparable to viremia titers found in natural hosts) coincided closely with the period of most rapid blood uptake in both larval and nymphal ticks. If the strain of ticks used in this study is representative of field populations, it can be concluded that *H. truncatum* is not a likely vector of RVF virus in nature.

In conducting the research described in this report, the authors adhered to the *Guide for the Care and Use of Laboratory Animals* as promulgated by the National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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